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The phenological stage of rice growth determines anaerobic ammonium oxidation activity in rhizosphere soil

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ABSTRACT

Anaerobic oxidation of ammonium (anammox) plays an important role in nitrogen (N) loss from agricultural systems. Recently, the rice rhizosphere was demonstrated to be a hotspot for anammox, yet the dynamics of anammox activity and the distribution of anammox bacteria in rhizosphere soil at different phenological stages of rice growth are still unknown. In this study, the activity, diversity and abundance of anammox bacteria in both rhizosphere and bulk soils were investigated over the entire rice growth season. From tillering to ripening stage, significantly higher anammox bacterial abundance was detected in rhizosphere soils compared to bulk soils. The rhizosphere soils also had significantly higher anammox rates at tillering and booting stages (0.71 and 0.32 nmol N g^{-1} dry soil h^{-1}, respectively) compared to bulk soils. The anammox rate in rhizosphere soil was positively correlated to the concentrations of NO_x^- (total of nitrate and nitrite) and acetate. The abundance of anammox bacteria was significantly correlated with the concentration of succinate in rhizosphere soils. A total of five anammox genera of *Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Scalindua* were detected, with *Brocadia* predominating in all examined samples. The distribution of anammox bacteria in rhizosphere and bulk soils varied with phenological stages. Statistical analysis indicated that C/N ratio, formate, citrate and ammonium were key factors influencing the composition of anammox bacteria. Variations in activity, abundance and distribution of anammox bacteria in rhizosphere were observed over the phenological progression, demonstrating that the root exudates might be influential for the anammox process. This study implies that future efforts in estimating the rate of anammox should consider the temporal variation during plant life cycles.

1. Introduction

Anammox, the oxidation of ammonium coupled to the reduction of nitrite with N_2 as the end-product under anaerobic conditions, greatly contributes to the loss of fixed nitrogen to the atmosphere in terrestrial ecosystems (Humbert et al., 2010; Shen et al., 2013; Zhu et al., 2011a). Agricultural fields with intensive application of mineral fertilizers have been reported as ideal habitats for anammox bacteria (Shen et al., 2013; Yang et al., 2015). Broad distribution of diverse anammox bacteria has been detected in agricultural soils (Zhu et al., 2011a; Shen et al., 2013; Wang and Gu, 2013), and a considerable proportion of N_2 production in paddy soils in Southern China could be attributed to the anammox process based on incubation studies (Yang et al., 2015).

Rhizosphere soils are important in N cycling, which has been largely overlooked in the past. Our previous work demonstrated the presence of anammox bacteria in rhizosphere soils, contributing higher N_2 production in comparison to bulk soils at the tillering stage of rice growth (Nie et al., 2015). However, the dynamics of the activity and diversity of anammox bacteria in

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rhizosphere soils over the entire growth period of rice remain unknown. Root exudates have been suggested to play a critical role in shaping the microbial community in soils (Lugtenberg et al., 2002; Hartmann et al., 2008; Shi et al., 2013). Also shifts in diversity and abundance of microorganisms (e.g. methane oxidizing bacteria) during rice growth have been observed (Eller and Frenzel, 2001; Dianou et al., 2012), which could be partially attributed to the shift in the quantity and quality of root exudates during the growth of rice (Badri and Vivanco, 2009; Chaparro et al., 2013).

We hypothesize that (i) the diversity and activity of anammox bacteria are different between the rhizosphere and non-rhizosphere zones in a fertilized paddy soil, and shift over the growth period of rice plants; and (ii) the quantity and composition of root exudates, especially the low molecular weight organic acids (LMWOAs), influence the rate and distribution of the anammox during various phenological stages. To address these hypotheses, we set up a pot experiment where rhizosphere and bulk soils were collected at different phenological stages of rice growth. The activity, abundance and composition of anammox bacteria were investigated using a ^{15}N-tracing experiment, qPCR and clone library analysis. The secretion activity of the rice root, and the composition of root exudates and soil chemical properties were measured to determine the factors influencing the anammox process in rhizosphere and bulk soils.

2. Materials and methods

2.1. Soil and pot experiment

The paddy soil (quaternary red clay, 0–20 cm) was collected from Taoyuan (28°55′15″ N, 111°27′35″ E), Hunan province of China on November 22, 2010. The characteristics of soil were listed as follows: pH (1: 2.5 soil/MilliQ water), 5.17; soil organic carbon (SOC), 47.7 g kg^{-1}; total nitrogen (TN), 2.68 g kg^{-1}; available phosphorus (AP), 5.36 mg kg^{-1} and available potassium (AK), 56.8 mg kg^{-1}. The soil sample was air-dried and sieved through a 2 mm mesh before pot experiment. Basic fertilizers (urea-N, 250 mg kg^{-1}; P as calcium superphosphate, 60 mg kg^{-1}; K_2O as KCl, 100 mg kg^{-1}) were well mixed with soil at the beginning of the experiment. No more fertilizers were applied during the growth periods. Each pot and rice seeds (cv. Xiangzaoshan 45) were pre-incubated as previously described (Nie et al., 2015). Rhizo-bag, allowing smaller molecular substrates to penetrate but prohibiting penetration by roots, was used to separate rhizosphere and bulk soils (Nie et al., 2015). The rice plants were incubated in greenhouse with natural illumination and humidity over the growth period in flooded condition with the temperature at 30 ± 2 °C during the day and 25 ± 2 °C in the night. The rhizosphere soils, bulk soils, rhizosphere pore water, bulk pore water and plants were sampled at each phenological stage (seedling, 12 d; tillering, 37 d; booting, 62 d; flowering, 82 d and ripening stage, 122 d) of rice growth. The pore water of rhizosphere and bulk soils were collected using one porous plastic soil moisture sampler (Rhizon SMS, Rhizosphere Research Products, Wageningen, Netherlands). The soils were divided into two parts. One part was used for analysis of anammox activity and chemical properties such as pH, moisture, NH_4^+ and NO_x^-, the other part was freeze-dried for genomic DNA extraction, SOC, TC and TN measurement. Analysis was carried out on soil samples from three separate pots at the same phenological stage.

2.2. Chemical analytical procedures

Soil pH was determined at a soil/MilliQ water ratio of 1:2.5 with a pH analyzer. The NH_4^+ and NO_x^- of soils were extracted with 2 M KCl at a soil/KCl ratio of 1:10 and filtered through a 0.22 μm hydrophilic PEPT needle filter (ANPEL, Shanghai, China). Subsequently, the filtrates were analyzed by a flow injection analyzer (FIA QC8500, Lachat, America). For the determination of TC, TN and SOC, the soils were sieved through a 0.15 mm mesh. The TC, TN and SOC were analyzed using a C/N analyzer (Vario MAX C/N, Germany) and a total carbon analyzer (TOC-V CPH, SHIMADZU, Japan), respectively. AP and AK were determined as previously described (Bao, 2000a, 2000b). Moisture of soil was assessed gravimetrically by drying the soil at 105 °C for 16 h. All samples were analyzed in triplicate.

2.3. Measurement of organic acids

The rice roots were sampled for LMWOA determination in the pot experiment. The soils around the rice roots were gently washed off with tap water, and the roots were washed finally using MilliQ water. As every care was taken, this procedure facilitated the removal of soil without injuring the roots. A 500 mL brown glass bottle with 300 mL MilliQ H_2O was used to collect root exudates from individual plant. After about 6 h extraction (Lu et al., 2007), the liquid was collected and filtered through a 0.22 μm hydrophilic PEPT needle filter (ANPEL, Shanghai, China). The collected filtrates and the pore water of rhizosphere and bulk soils sampled above were used for analysis of LMWOAs by ion chromatograph (ICS-3000; Dionex).

2.4. Anammox and denitrification rate measuring

The rates of anammox and denitrification in rhizosphere and bulk soils were measured by ^{15}N stable isotope tracing technique as previously described (Yang et al., 2015) with some modifications. About ~3.5 g soil was transferred into a 12 ml gastight vial (Exetainer, Labco, High Wycombe, Buckinghamshire, UK), which were then filled totally with N_2-purged overlaying water (Wang et al., 2012a) and sealed with a gastight lid. The resulting slurries were pre-incubated for 1 day to remove residual NO_x^- and oxygen according to our preliminary experiment when both NO_2^- and NO_3^- were under the detection limit of ion chromatograph (0.05–0.1 ppm and 0.075–0.1 ppm for NO_2^- and NO_3^-, respectively). Subsequently, 100 μL of N_2-purged stock solution of each isotopic mixture: (1) $^{15}NH_4^+$ (^{15}N-$(NH_4)_2SO_4$, ^{15}N at.%: 99.14), (2) $^{15}NH_4^+ + {}^{14}NO_3^-$, and (3) $^{15}NO_3^-$ (^{15}N–KNO_3, ^{15}N at.%: 98.15), was injected through the stopper of each vial, resulting in a final concentration of about 100 μM N, which was added in access based on the maximum TN concentration (*ca.* 70 μM N) detected in all collected samples. The incubations were performed at a temperature of 25 ± 1 °C. Time course incubations were carried out in duplicate (time points 0, 3, 6, 12, and 24 h). $ZnCl_2$ solution (200 μL, 7 M) was added at each time point during the incubation in order to stop microbial activity (Zhu et al., 2011b). The N_2 in slurries was determined within 24 h after incubation. For the measurement of N_2 production, 2 mL headspace water sample of gastight vial was transferred into a new gastight vial filled with analytical grade (99.99%) helium using a 5 ml syringe in a condition without N_2. The vials were shaken violently for about 1 min and stored upright at room temperature to allow N_2 to equilibrate. The content of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ in headspace was then analyzed by Isotope Ratio Mass Spectrometers (MAT253 with Gasbench II and autosampler (GC-PAL), Bremen, Thermo Electron Corporation, Finnigan, Germany) using air with constant $^{15}N/^{14}N$ as standards. The equations and their explanations were described by Thamdrup and Dalsgaard (2002).